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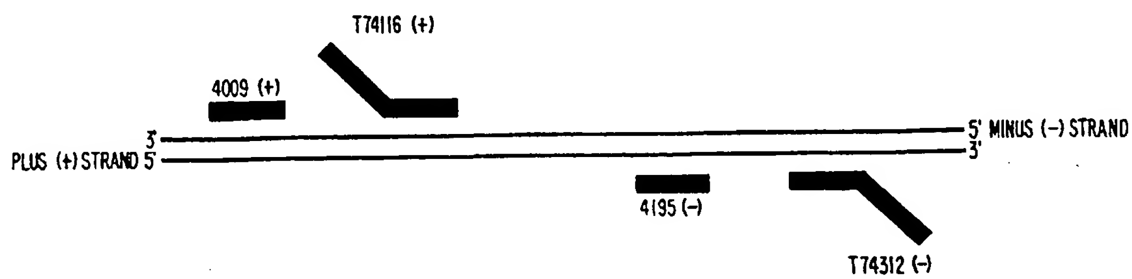

(57) Abstract

A method for amplification of a nucleic acid strand in a test sample. The method includes contacting the nucleic acid strand from the test sample simultaneously with at least three oligonucleotide primers. At least one primer is a promoter-primer, and at least one other primer is complementary to the nucleic acid strand, and one other primer is complementary to a strand complementary to the nucleic acid strand. The method further includes contacting the nucleic acid strand and primers with one or more proteins having RNA-directed and/or DNA-directed DNA polymerase activities, an RNA polymerase activity, and an RNAse H activity under primer-extension conditions to allow amplification of a target region in the nucleic acid strand at essentially constant temperature.

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DESCRIPTION

Methods for Enhancing Nucleic Acid Amplification

This invention relates to amplification of nucleic acid strands using a DNA polymerase and an RNA polymerase at essentially constant temperature.

Background of the Invention

The ability to detect specific nucleic acid sequences has afforded many practical benefits in areas such as genetic research, clinical diagnostic testing, forensic sciences, archaeology, etc. In many cases, the sequence of interest might be present at a level much too low to detect directly, even using probes with very high specific activity labels. In recent years, strategies have been devised for efficiently generating new copies of target sequences, including very powerful exponential amplification methods, which make it easier to accurately detect the presence of very low target levels.

One such method is the polymerase chain reaction (Mullis et al., U.S. Patent 4,683,202) in which a reaction mix of primers, substrates, DNA polymerase and analyte nucleic acid is subjected to *n* cycles of heating to a temperature sufficient for denaturing double-stranded nucleic acids and cooling to a temperature at which primer annealing and extension can occur. This reaction is well understood to have a maximum amplification factor of 2^n since each strand of a target sequence can be copied into (at most) one new complementary strand during each cycle.

The performance of target-specific amplification has been augmented by performing two or more successive amplification reactions in which the target region defined by the primers used in the subsequent rounds is contained within the target amplicon generated by primers used in the previous round. Even if the amplification of a desired target is inefficient in the first round because

of co-amplification of non-target sequences, the target amplicons that are generated should have a selective advantage for further amplification by the next primer set since non-target amplicons are usually not more effective templates for further amplification by the nested primer set than other non-target sequences present. This strategy has been used to improve the ability of amplification methods such as PCR (Conway et al., J. Acquired Immune Def. Syndromes 3:1059 (1990); Matsumoto et al., J. Virol. 64:5290 (1990); Garson et al., Lancet 336:1022 (1990); and NASBA (Kievits et al., J. Virol. Methods 35:273 (1991)) to detect very low target levels.

The amplification method of Kacian et al. PCT-/US90/03907 depends on multiple enzyme activities (i.e., including RNA polymerase, DNA-directed DNA polymerase, RNA-directed DNA polymerase, and RNase H). Although it is possible to provide these activities by contacting the other reactants with separate enzymes possessing one each of these activities, a preferred configuration uses a single enzyme, reverse transcriptase, as the principal source of the last three activities listed above. For example, one embodiment of this method employs RNA polymerase from coliphage T7 and reverse transcriptase from Moloney murine leukemia virus (MuLV) in a reaction which supports amplification extents of up to 10^{12} fold or more.

The rate of accumulation of products is much more complicated for such an asynchronous, continuous amplification process but is calculable based on straightforward physical properties of the reaction components.

The exponential accumulation of amplification products does not proceed indefinitely in any of these methods. The rate per time of new copy production reaches a maximum as the enzymes present become saturated by the number of existing templates available to be copied. Thus, the system changes with time to a linear, rather than exponential, rate of accumulation. Ultimately the amount of product made is limited by the number of mole-

cules of those substrates, such as primers and nucleosides, which are physically incorporated into amplification products.

Summary of the Invention

This invention relates to a significant improvement of the process described by Kacian et al. In particular, it relates to methods for improving the sensitivity of the process, i.e., the ability to amplify desired target sequences that are present in extremely small numbers.

Applicant believes that the most significant and prevalent obstacle to achieving maximum sensitivity is competition for reaction components by amplification of non-target sequences. Although primer annealing and extension should be most efficient on target sequences which are highly complementary to the primer, the possibility that a primer can complex with, and be extended upon, a sequence with only a few bases of complementarity to the 3′ end of a primer is thermodynamically predictable and empirically known in the art. Even if the frequency per site of non-target initiation is low, the number of non-target bases in a reaction is usually much greater than the number of targeted bases complementary to the primers used to select the target sequence. Since a primer is physically incorporated into the initiation product, subsequent complementary copies can be very active templates for further amplification even though the original progenitor sequence scarcely resembled the desired target.

The relative specificity of initiation by different primer sequences can vary over quite a great range and while the specificity cannot reliably be predicted based on sequence alone, it is possible to identify preferable sequences by routine experimentation. However, the considerations described above imply that for even the best primers, the potential for interference by non-target initiation becomes increasingly severe as the number of

target molecules is reduced since it becomes more probable that at some point early in the reaction, the population of non-target amplicons will be larger than the population of target-specific amplicons. The difference between these population sizes can be amplified exponentially as the reaction proceeds, and it is possible in such a case that the depletion of reaction components by non-target amplification causes the reaction to slow or stop before the target-specific product reaches detectable levels.

It is well known in the art that the stability of a base-paired complex between two nucleic acid sequences decreases as the temperature is increased. This usually results in an apparent increase in the specificity of detectable hybridization since hybrid thermal stability depends on the extent and continuity of base-pairing. Improvements in the yield of target-specific amplicon and reduction in the accumulation of non-target products were observed when the availability of a thermostable DNA polymerase made it possible to use higher reaction temperatures for PCR (Saiki <u>et al</u>., <u>Science</u> 239:487 (1988)). Flexibility in selecting a reaction temperature has simplified effective optimization of PCR systems by routine experimentation (Rychlik <u>et al</u>., <u>Nucleic Acids Res.</u> 18:6409 (1990)). However, development of systems for reliable detection of very low target levels (<u>e.g.</u>, <50) remains challenging.

Although raising the temperature reduces the lifetime of base-paired complexes once formed, higher temperatures also increase the rate of collisions between molecules to form potentially extensible complexes. Applicant has found that the amount of non-target priming increased at temperatures both above and below a measured optimum. Thus, it is rare that one can expect to achieve absolute specificity for the desired target based on controlling the temperature alone.

Other strategies have been described for enhancing the specificity of primer extension including use of

chemical denaturants and single-stranded binding proteins. Although these strategies have been useful in some cases, consistently favorable conditions have not been described.

At this time, thermostable variants of reverse transcriptase which retain all three activities noted above are not known. Thermostable RNA polymerases have been described but none as yet having a promoter specificity as well-characterized as T7 RNA polymerase. Methods are known in the art to screen for, select for, and/or engineer enzyme variants with desirable properties, including thermostability, but the methods disclosed herein afford another solution to the challenge of enhancing initiation specificity and, consequently, the sensitivity of target amplification. These methods have been especially effective in compositions having a small number of target sequences in the presence of a vast excess of non-target nucleic acids, and furthermore, can be employed together with elevated temperature treatments.

The methods disclosed herein employ the concept of amplicon nesting, but are significantly different from previously described strategies in which a portion of a reaction run with the first primer set is transferred to a new reaction containing the second primer set. In the methods described herein, all the primers delimiting the nested amplicons can be combined in a single reaction such that serial transfer of products to a new reaction is unnecessary, and furthermore, the best mode is apparently favored by a dynamic coordination among their activities.

Increasing the number and types of primers present in the mixture does significantly increase the potential for various side reactions, including those leading to competitive, non-target amplification. The extra primers added also have the potential to interfere with the desired normal function of the principal primer set. Therefore, it was unexpected that we could identify conditions wherein the degree of enhancement was not only unequivocal but of such a dramatic extent. Note that the

method functions through a continuous process and does not require or employ any heat treatments to thermally denature double-stranded primer extension products.

Thus, in a first aspect, the invention features a method for amplification of a target sequence in a nucleic acid strand in a test sample. The method includes contacting the nucleic acid strand from the test sample simultaneously with at least three oligonucleotide primers. At least one primer is a promoter-primer (i.e., having a primer region complementary to the nucleic acid strand or its complement, and another region, 5′ of the primer region, recognized in its double-stranded form by an RNA polymerase), and at least one other primer is complementary to the nucleic acid strand, and one other primer is complementary to a strand complementary to the nucleic acid strand. The method further includes contacting the nucleic acid strand and primers with one or more proteins having RNA-directed and/or DNA-directed DNA polymerase activities, an RNA polymerase activity, and an RNAse H activity under primer-extension conditions to allow amplification of a target region in the nucleic acid strand at essentially constant temperature.

A "test sample" includes any clinical, agricultural, or environmental sample which may or may not be pretreated to make the nucleic acid strand available for hybridization with the primers. Such a strand is not amplified by other methods prior to the first contacting step described herein. That is, the method of this invention can be used directly to amplify a nucleic acid within such a sample. No prior amplification by PCR or the method of Kacian et al. is necessary. The method essentially features the method of Kacian et al., but with an additional primer provided to significantly and unexpectedly enhance target amplification at the expense of non-target amplification.

By "oligonucleotide" is meant to include a nucleic acid molecule with at least two nucleoside residues joined through a phosphodiester linkage, or an analog of a

phosphodiester linkage known in the art. The nucleotide base moiety of the oligonucleotide may be adenine, guanine, cytosine, thymine, uracil, or other naturally-occurring or synthetic base derivatives, especially those which can complex with a complementary base in another nucleic acid sequence to participate in a double-stranded nucleic acid structure. The sugar moiety may be ribose, deoxyribose, or other derivatives or modified forms of these structures. Many derivatives of the phosphodiester moiety are known in the art and can be used in the invention. An oligonucleotide may also contain domains or residues which are not nucleosides and which might be used, e.g., as a linker to a label or solid support, or to provide other functionality. Oligonucleotides can be synthesized chemically or by use of nucleic acid polymerases, or processed from naturally occurring nucleic acids, by many methods which are well known in the art.

By "primer" is meant a molecule which can be used by a nucleic acid polymerase as a receptor for covalent addition of a suitable nucleoside-5'-phosphoryl (or equivalent) residue. It is convenient to use an oligonucleotide with an extensible 3' end as a primer since it is straightforward to control the sequence of the primer and thus influence the polymerase to copy desired target sequences which are adjacent to sequences complementary to the primer; however, other molecules with priming activity, such as some proteins, are known.

By "promoter-primer" is meant a primer which also has sequence or structural properties which can interact with an RNA polymerase to cause the RNA polymerase to transcribe a desirable template. The promoter-primers used in the examples herein are oligonucleotides which consist of sequences known to be part of an effective promoter for T7 RNA polymerase linked to sequences which are complementary to desired targets in, e.g., the HIV genome. Other promoter sequences are known and can be used including promoters for T3 RNA polymerase and SP6 RNA polymerase.

Other strategies can also be employed to promote relatively specific transcription and are intended to be covered by this definition of promoter-primer. For example, an RNA oligonucleotide which is hybridized to a DNA template, especially in a heterotriplex structure (sometimes called an R-Loop) resembling a nascent RNA transcript, can be extended by an RNA polymerase to yield an RNA complement of a desired target template.

By "target region" or "amplification target" is intended to mean a sequence of consecutive nucleotide residues which one desires to amplify by duplication of this sequence or its complement by successive rounds of nucleic acid polymerization. It is not necessary to know the nucleotide sequence of the entire target region but it is helpful to know enough sequence to design at least one complementary primer and a sequence which can be used for specific detection of amplification products, such as by hybridization with a labeled complementary probe.

The phrase "non-target nucleic acid" includes all sequences which are not contained within such a desired target region. These might include, for example, other sequences present on the same genome as the target region, nucleic acids from other genomes or their gene products present in the reaction, such as from a host cell or from environmental contaminants, and nucleic acids deliberately added to the reaction, such as the primers.

In preferred embodiments, the nucleic acid strand is a single-stranded DNA strand or converted to single-strands by denaturing double-stranded DNA; the nucleic acid strand and primers are first contacted at 60°C or above with an enzyme having DNA polymerase activity active at 60°C or above; the second contacting step is at 42°C or above in the presence of a reverse transcriptase and an RNA polymerase; four primers are used in the first contacting step; at least one primer is provided at a concentration different from one other primer; all enzyme activities are provided by a reverse transcriptase and an

RNA polymerase; but its enzyme activities may be supplemented by an RNAse H having no DNA polymerase activity; the DNA polymerase lacks 5'-3' exonuclease activity, and is derived from the DNA polymerase I of a Bacillus species; e.g.: of the species Bacillus stearothermophilus or Bacillus caldotenax; the two outside primers hybridize to said nucleic acid strand or its complement at most 2000, 500, or 350 bases apart; and one primer is provided at a concentration between 1 and 10 μM and another said primer is provided at a concentration between 10 and 50 μM.

In other preferred embodiments, two primers are plus-sense primers and the inside plus-sense primer is a promoter-primer; or two primers are minus-sense primers and the outside minus-sense primer is a promoter-primer. References to position and polarity are intended to have the meanings described below in reference to the structures in Fig. 1 and do not depend on polarity designations which might be conventional for the genetic system in which a target region is found. Thus, T74116 and 4195 in Fig. 1 are considered herein to be inside primers; T74312 and/or 4009 are considered to be outside primers. Of the possible amplicons which can result from this array of primers, it is expected that the sequence within the target region delimited by the inside primers will amplify to the greatest extent because amplification products which are delimited by one or both outside primers are targets for annealing by the complementary inside primer but the converse is not necessarily true. Therefore, the target region delimited by the inside primers in Fig. 1 is considered to be the principal target region, and T74116 is an example of the principal promoter-primer.

The sense of the endogenous target region which is complementary to the principal promoter-primer is defined as negative or minus sense, as are other nucleic acids present which have the same sequence sense as the minus target strand. Thus, the principal promoter-primer is

defined as positive or plus sense, as are other nucleic acids present that are complementary to the minus sense nucleic acids. It will be apparent to those skilled in the art that these assignments are valid even if the native form of the endogenous template containing the target region is a single-stranded nucleic acid molecule (e.g., RNA) since this strand comprises sufficient information to uniquely specify a complementary strand, and such a complement can be synthesized by the reaction components.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof and from the claims.

Description of the Preferred Embodiments

The drawings will first briefly be described.

Drawings

FIG. 1 is a diagrammatic representation of the position of primers relative to the structure of the pol1 target region;

FIG. 2 is a diagrammatic representation of amplification Initiation Methods, IM1, IM2 and IM3 protocols.

FIG. 3 is a diagrammatic representation showing a possible scheme for initiation by extension of outside T7 primer; and

FIG. 4 is a diagrammatic representation showing potential strand displacement activity of T74116 primer extension product by subsequent extension of 4009 primer which may help make target-specific initiation more efficient.

Examples

The following are non-limiting examples of the present invention. Those in the art will recognize that variations to these examples are within the scope of the

appended claims. In particular the specific amounts of reagents and their proportions, and the specific enzymes and nucleic acids used, can be varied to allow amplification of any chosen target. In these examples, certain terms are used as follows.

"Initiation" refers to the process by which an endogenous template sequence is copied or converted into a form which can be transcribed efficiently to yield RNA copies of the target region or its complement (whether this is a desirable target region or not). In the amplification method of Kacian et al., initiation at a particular target is complete when such an RNA product is capable of participating as a template in a cycle of reaction steps, which can lead to de novo formation of a template that can be transcribed to yield an essentially similar RNA molecule. (This RNA molecule may not be identical in sequence to its precursors but retains at least enough sequence similarity to be amplified further).

"Amplicon" refers to a nucleic acid that is a product of one of the reactions in the amplification cycle and which retains the ability to serve as a template for further amplification.

"Pre-initiated template" is used to designate a nucleic acid that possesses the properties of an amplicon, i.e., it can serve as a template for one of the reactions in the amplification cycle without first participating in one of the initiation reactions. A pre-initiated template may indeed be an amplicon product of a prior amplification reaction, or might be constructed synthetically as an experimental model of amplicon activity by methods such as PCR, chemical synthesis or cloning.

As suggested above, the amplification reaction can be perceived as having two phases, one phase including those reaction steps causing the endogenous template to be copied or converted into a functional amplicon, and the second phase including those steps that are part of the inherently cyclical amplification process. The intermedi-

ates and products of the amplification steps are essentially similar regardless of the original endogenous template, but the initiation steps used depend on the properties of the endogenous template. Various initiation strategies for the target amplification method of Kacian et al., *supra*, have been described previously; some of them are described briefly here for convenience and shown diagrammatically in Fig. 2.

Referring to Fig. 2, Initiation Method 1 (IM1) refers to an initiation method in which the endogenous template is DNA. Under conditions allowing a promoter-primer to anneal to a complementary target, a DNA polymerase activity is added to synthesize a complement to the target template by extension of the promoter-primer. The reaction is heated (e.g., at 95°C) to denature the double-stranded DNA product and cooled to a temperature which allows annealing of a second primer to a complementary sequence on the newly synthesized extension product. When suitable enzymes are added (e.g., RNA polymerase, reverse transcriptase and, optionally, RNase H), the second primer can be extended by DNA polymerase activity to produce a double-stranded copy of the target region linked to a promoter, and thus an active template for the RNA polymerase.

Initiation Method 2 (IM2) refers to an initiation method in which the endogenous template is DNA. A single addition of enzymes (including RNA polymerase, reverse transcriptase and, optionally, RNase H) is sufficient to yield effective initiation even if the reaction is not heated to denature the initial primer extension products. Competent amplicons are generated in the reaction via intrinsic processes in the isothermal reaction.

Initiation Method 3 (IM3) refers to an initiation method in which the endogenous template is RNA. The reaction can be assembled and receive a single enzyme addition (including RNA polymerase, reverse transcriptase and, optionally, RNase H). The double-stranded product of

the initial extension of the promoter-primer is an RNA/DNA hybrid. The RNA strand is a substrate for the RNase H activity present and can be degraded to yield a single-stranded copy of the promoter linked to the target region, which in turn is a template for extension of the second primer as described above.

The terms "reaction failure" or "amplification failure" as used herein are not meant to imply that amplification failed to occur but simply that copies of the desired target sequence were not detectable among the products. This may indicate the absence of the desired target among the analyte nucleic acids. This might also result from target-specific initiation or amplification which was not sufficiently effective. For example, target-specific initiation might be ineffective even though many specific initiation events occurred if initiation on non-target sequences yielded excessive competitive amplicons. As will be shown in the examples below, the present invention provides sufficient improvement over existing methods to allow detection of as few as 1-5 copies of a target nucleic acid within a sample without requiring additional heating steps to denature reaction intermediates.

General Methods

The procedures described in this section, or slight variations thereof, were used in most of the examples described below. Exceptions and modifications are detailed in each example.

The following is an example of an IM2 amplification reaction.

1) A solution containing the following components was prepared and dispensed in a volume of 25 μl:

200 mM Tris·HCl (pH 8.0 at about 20-25°C)
70 mM $MgCl_2$
8 mM spermidine
0.4 mM deferoxamine mesylate
25 mM each GTP & ATP
10 mM each UTP & CTP
0.8 mM each dATP, dGTP, dCTP, dTTP
0.6 μM T74116 promoter-primer
1.2 μM 4195 primer
20% (v/v) glycerol

The primers used in the examples are shown diagrammatically in the figures. They have the following sequences: SEQ. ID NO. 1 (4009): 5-'ATTCCCTACAATCCCCAAAGTCAA-3'; SEQ. ID NO. 2 (T74116): 5'-[AATTTAATACGACTCACTATAGGGAGA] CAAATGGCAGTATTCATCCACA-3'; SEQ. ID NO. 3 (4195): 5'-GTTTGTATGTCTGTTGCTATTAT-3'; and SEQ. ID NO. 4 (T74312): 5'-[AATTTAATACGACTCACTATAGGGAGA] CCCTTCACCTTTCCAGAG-3'. (The promoter sequences are shown in brackets, other promoter can be used in this invention.) The HIV sequences of T74116, 4195 and T74312 were disclosed previously (McDonough et al, U.S. Patent Application Serial No. 08/040,745 hereby incorporated by reference herein).

2) To this mixture was added 50 μl of a sample containing the nucleic acids to be analyzed. Model reference system samples contained 1 to 10 μg of purified human white blood cell (WBC) DNA in 80 mM potassium acetate. WBC DNA can be prepared by a variety of well-known methods (See, Maniatis et al., Molecular Cloning, a laboratory manual, Cold Spring Harbor Press, Cold Spring Harbor, NY, 1982). Alternatively, 50 μl of a hydrolyzed WBC lysate, prepared as described in Example 4, was used. Reactions received 5 μl of water if negative controls, or 5 μl containing a known amount of purified,

clonedHIV nucleic acid for testing amplification performance.

3) The mixture was heated to 95°C and maintained at this temperature for 5 min. It was then transferred to 42°C and allowed to cool to this temperature.

4) Twenty μl of a solution containing 800 U Moloney MuLV reverse transcriptase (RT) and 400 U T7 RNA polymerase was added in a solution comprising 50 mM Tris·HCl (pH 8.0), 10 mM potassium acetate, 100 mM N-acetyl-L-cysteine and 20% (v/v) glycerol.

5) This was mixed briefly and incubated at 42°C for 2 hr.

6) The formation of amplification product containing the intended target sequence was determined using a specific hybridization procedure. For all experiments described herein the hybridization protection assay (Arnold et al., Clin. Chem. 35:1588 (1989) and PCT/US88/03195) was used.

Unless specified otherwise in the examples below, the pol1 primers were used in the IM2 experiments at the concentrations listed above (i.e., 15 pmol T74116 and 30 pmol 4195 per 100 μl reaction). When gag11 primers were used, T7811 and 872 were added at 30 pmol each per 100 μl reaction.

Strategies for enhanced initiation effectiveness were tested using the following modifications of the basic IM2 procedure:

1a) A mixture of reaction components was prepared as described in Step 1 in the IM2 procedure. Optionally, additional oligonucleotides were added as outside primers, e.g., 3 pmol each per reaction of 4009 and T74312 for pol1 amplification.

2a) This mixture received 50 μl of a sample containing the nucleic acids to be analyzed. Model reference system samples contained 1 to 10 μg of purified human WBC DNA in 80 mM KOAc. Alternatively, 50 μl of a hydrolyzed WBC lysate, prepared as described in Example 4, was used. Reactions received 5 μl of water for negative controls, or 5 μl containing a known amount of purified, cloned HIV nucleic acid.

3a) The mixture was heated to 95°C and maintained for 5 min. It was then transferred to 60°C and allowed to cool to this temperature.

4a) Optionally, 10 μl of a solution containing a thermostable DNA polymerase was added in a solution comprising 50 mM Tris·HCl (pH 8.0), 10 mM potassium acetate, 100 mM N-acetyl-L-cysteine and 20% (v/v) glycerol. Enzymes tested and desirable properties thereof are described in the examples below.

5a) The reaction was mixed briefly and incubated at 60°C for 10 min.

6a) The reaction was transferred to 42°C and allowed to cool to this temperature.

7a) 10 μl of a solution containing 800 U Moloney MuLV reverse transcriptase and 400 U T7 RNA polymerase was added in a solution comprising 50 mM Tris·HCl (pH 8.0), 10 mM potassium acetate, 100 mM N-acetyl-L-cysteine and 20% (v/v) glycerol.

8a) The reaction was mixed briefly and incubated at 42°C for 2 hr.

9a) The formation of amplification product containing the intended target sequence was determined using a specific hybridization

procedure such as the hybridization protection assay (Arnold et al., supra).

The outside primers used (if any) and their concentrations are described in each of the examples.

We found that the most valuable indicator of initiation effectiveness was the frequency of reaction failures for a particular template level rather than the extent of amplification in individual reactions. Therefore, for each condition tested, experiments were set up with multiple replicate reactions so that improved initiation effectiveness could be identified by a statistically significant decrease in the failure frequency. Furthermore, the geometric means (G.M.) of the signals for the replicate reactions correlated well with initiation effectiveness and are shown for most of the examples.

The HIV templates used in experiments described in the Examples were purified by standard methods from *Escherichia coli* containing plasmid clones of HIV sequences (see for example Maniatis et al., supra). In experiments specifying BH10 DNA, the template was a purified double-stranded linear DNA having essentially the 8932 nucleotide sequence described in Genbank as HIVBH102 (Accession No. M15654) plus the complementary strand. Other experiments used a linearized plasmid DNA (pUCHIV) comprising the *gag* and *pol* genes of BH10 in a standard pUC cloning vector. Both templates had virtually identical template activity per molecule in side by side comparisons.

After purification, the concentration of DNA in these preparations was determined by measuring the amount of 260 nm ultra-violet light absorbed by samples of each preparation (A_{260}). The nucleotide sequence, and thus the length, of each of these DNA species is known. The molar concentration for such a preparation was determined by applying standard conversion factors: mass concentration of double-stranded DNA = 50 μg ml^{-1} A_{260}^{-1}; molecular weight of double-stranded DNA = length(bp) × 650 g mol^{-1} bp^{-1}. A stock

solution of template DNA at a concentration ≥10^8 templates per 5 μl (33 pM) was divided into separate aliquots and frozen. For each amplification experiment an aliquot of template was thawed and serially diluted to the desired working concentration (e.g., 5 templates per 5 μl) for addition to reactions. The thawed aliquots and dilutions were discarded after each experiment.

Example 1: Initiation Effectiveness

To assess quantitatively the effect of various reaction parameters on initiation effectiveness, it was desirable to develop methods to discriminate between changes in amplification effectiveness and in initiation effectiveness in response to a given variable. This was necessary because, for example, we found that conditions favoring optimum amplification performance were not necessarily the conditions which yielded optimum initiation effectiveness. One way we accomplished this was to add pre-initiated templates to reactions as an indicator of the intrinsic amplification performance of various reaction compositions or treatment scenarios and to compare these results with the amplification resulting from addition of a native target sequence.

Using this method, it was possible to determine how rapid and extensive the initiation of target-derived amplicons must be to out-compete the amplification of non-target sequences. An amplification time course was performed in which reactions were assembled according to various desired test conditions but without any target template. At various times after the reaction was started by addition of the RT and RNA polymerase enzymes, template was added and the resulting final amplification extents determined as described below:

1) A mixture was prepared containing the following components, and 85 μl of the solution was dispensed into each reaction tube. The concentra-

tions listed refer to the respective concentrations in the completed 100 μl reaction.

50 mM Tris·HCl (pH 8.0 at room temperature)
17.5 mM $MgCl_2$
5 mM dithiothreitol
2 mM spermidine
6.25 mM each GTP & ATP
2.5 mM each UTP & CTP
0.2 mM each dATP, dGTP, dCTP, dTTP
0.3 μM each T74116 promoter-primer and 4195 primer
3 μg human WBC DNA

2) The reactions were heated to 95°C for 7 min, transferred to 37°C and allowed to cool to this temperature for 5 min.

3) Moloney MuLV reverse transcriptase (600 U) and T7 RNA polymerase (400 U) were added to each reaction in 10 μl of buffer (10 mM Tris HCl (pH 8.0), 10 mM potassium acetate and 5 mM dithiothreitol).

4) At various times after enzyme addition, either 100 copies of single-stranded BH10 DNA (purified, cloned HIV DNA, previously denatured by boiling) or 10 copies of pre-initiated template were added to respective reactions in a volume of 5 μl. Three replicates of each time point and condition were processed.

5) After 2 hours the yield of target-specific amplification product determined by the hybridization protection assay (Arnold et al., supra).

Analysis of the geometric mean of the signals of three replicates for each condition shows that even ten (10) pre-initiated amplicons could not be amplified to detectable levels if the amplification biochemistry is allowed to proceed for as few as 10 minutes in the presence of non-target nucleic acids but the absence of target

nucleic acid. Furthermore, about ten (10) times more endogenous template was required to achieve an essentially similar time course of amplification as was observed for the pre-initiated template. The difference is explained by the immediate entrance of pre-initiated template into the amplification cycle whereas the non-target amplicons already present continue to accumulate exponentially during the time required for the native template to be copied via the initiation reactions into an amplification competent form. For each of these conditions, more than 90% of the target-specific amplification potential was lost within 3-5 minutes of adding the reverse transcriptase and RNA polymerase to begin the amplification process. The extreme brevity of this window of opportunity for effective initiation was very surprising even though we had expected significant levels of non-target priming and initiation. The trends observed here, as well as in many comparable experiments, suggest that the inhibition was due to excessive depletion of essential reaction components by amplification originating from high levels of non-target initiation.

It has been possible to detect moderately low target levels in many cases using amplification systems developed by routine optimization of methods disclosed by Kacian _et al._, _supra_. For example, using the IM2 method and the pol1 primer set we were able to detect virtually every test sample which contained ≥50 HIV genomes and about 2/3 of the samples containing 20 HIV genomes. This performance reflects very powerful amplification, which would be more than adequate for most purposes. There are, however, cases in which even greater sensitivity is desired. HIV is one example of a pathogen whose nucleic acids might be present at a very low concentration in infected tissues such as whole blood. Reliable detection of HIV nucleic acids sometimes requires a significant sample size (_e.g._, WBCs from ≥ 0.1 - 1 ml blood or more) to ensure that at least one target sequence is present. The nucleic acid

extracted from such a sample might contain a single HIV genome in the presence of >20 μg of non-target DNA. The severely aggressive character of competitive non-target amplification as revealed in Example 1 makes it clear that detecting the target in such a sample was a very challenging goal and could not be expected by routine experimentation.

Example 2: Thermostable DNA Polymerase

This example demonstrates that significant increases in sensitivity can be achieved by application of the principles disclosed here. Samples **A** and **B**, shown in Table 1 were treated using the standard IM2 method as described under General Methods above, i.e. the samples were cooled from 95°C directly to 42°C and the reverse transcriptase/T7 RNA polymerase mixture was added to begin the reaction. Samples (**C-F**) were cooled from 95°C to 60°C, as described, received 2 U *B. stearothermophilus* (*Bst*) DNA polymerase each, and were allowed to incubate for 10 min. The samples were then allowed to cool to 42°C before receiving the reverse transcriptase/T7 RNA polymerase mixture. Each reaction received purified cloned HIV DNA (pUCHIV) diluted to an average of 5 templates per reaction.

Table 1

Bst	Outside Primers (3 pmol)			
	None	T74312	4009	4009 + T74312
	A			B
None	2,822 788,364 5,609 550,515 54,499 2,962 884,319 2,404 5,601 301,269	n.d.	n.d.	5,575 1,080,914 598,645 2,904 399,264 692,780 3,057 907,013 3,386 635,132
G.M.:	36,305			83,898
	C	D	E	F
2 U	2,684 21,699 500,660 2,685 222,122 157,526 518,992 318,567 736,861 2,896	894,475 1,007,288 10,027 914,272 897,114 923,988 942,281 962,413 963,703 78,465	996,174 573,620 933,090 230,777 982,900 701,584 802,113 939,987 3,605 1,100,968	1,053,438 925,349 985,495 981,515 953,186 1,000,703 1,011,202 1,013,977 958,185 1,040,630
G.M.:	63,108	464,691	436,989	991,645

The amount of target sequence generated is expressed in Tables 1 - 7 in relative light units (RLUs), a measure of the amount of signal obtained from the chemiluminescent label on the detection probe.

The RLU values for the negative control (no pUCHIV) for each of these reaction conditions (**A-F**) were: 2591, 3097, 2471, 3569, 3459 and 6030, respectively.

These results show that using either a high temperature initiation step with *Bst* polymerase (**C**) or including the outside primers even at 42°C (**B**) can each alone enhance initiation effectiveness. The most dramatic

enhancements were seen when the 60°C supplemental initiation step was performed in the presence of either of the outside primers (**D**, **E**), and the best condition included both outside primers as well as the 60°C supplemental initiation using *Bst* DNA polymerase (**F**).

EXAMPLE 3: PRIMER TITRATION

This example shows some of the surprising properties of the enhanced initiation systems, which make it clear that these enhancements were not obvious nor predictable from prior art.

The most effective concentration of outside primers was determined by titration. In this example, both the T74312 promoter-primer and the 4009 primer were included at equimolar levels as shown in Table 2. The experiment was also intended to determine if initiation enhancement was due primarily to the primer nesting, to the high temperature step alone, to the high temperature incubation in the presence of DNA polymerase, or to some combination of these factors. The reaction condition (**A**), which had no *Bst* polymerase and no outside primers, was executed using a standard IM2 initiation as outlined under General Methods above (i.e., no 60°C step). All the other samples received the 60°C incubation step whether or not *Bst* polymerase was included in the reaction.

Each sample shown in the table received an average of 5 molecules of pUCHIV DNA. A negative control was also done for each reaction condition; the RLU values for the negative controls were: 1481, 3073, 1888, 1579, 2150, 1685, and 2038, for **A-G**, respectively.

Table 2

Bst	Amount of T74312 & 4009			
	None	0.5 pmol each	1 pmol each	3 pmol each
	A	B	C	D
None	1,539 1,817 276,389 703,977 504,437 2,190 112,011 945,321 450,767 2,021	2,613 2,798 2,618 6,461 2,499 98,767 2,563 2,362 17,165 2,234	1,839 1,968 1,859 1,735 1,827 978,524 1,767 53,199 1,713 187,509	3,196 916,062 71,336 377,802 322,609 991,897 932,431 125,527 716,442 791,526
G.M.:	**47,460**	**4,611**	**7,585**	**264,500**
		E	E	G
2 U	n.d.	816,921 2,405 990,140 990,692 1,008,058 957,396 968,449 957,421 1,031,290 2,055	934,499 925,259 992,702 979,840 966,982 997,355 994,863 982,283 937,674 934,387	960,554 920,915 952,251 1,012,172 954,368 1,011,579 974,269 1,008,390 1,017,541 1,023,782
G.M.:		**285,949**	**946,198**	**982,999**

As described above, it is possible for the extra primers included in the reaction to inhibit target-specific amplification by promoting additional initiation on non-target sequences. This potential for interference with desired amplification by extra primers in the reaction is observed here, i.e. in the reactions with 0.5 or 1 pmol each outside primer in the absence of the higher temperature pre-initiation step (B, C). In contrast, 3 pmol of each outside primer (D) yielded significantly better initiation effectiveness than the standard IM2

initiation condition (**A**). The inclusion of the 60°C primer extension step (**E-G**) not only broadens the range over which the nested primer strategy is effective, but also, as in the previous example, is synergistic with the best outside primer conditions (**G**) to yield impressive initiation effectiveness.

EXAMPLE 4: CRUDE LYSATES

This example showed that the initiation enhancements were not only functional, but even more valuable, when applied to a crude lysate typical of a patient sample after appropriate processing. Because of the complex and variable chemical composition of such lysates, it is typical for at least some of the amplification processes to proceed less effectively in lysate than in systems with purified components. Therefore, signals are often lower and/or failures more likely than for comparable target levels in a reaction containing purified components such as the model reference system reaction.

1) Whole blood treated with EDTA as an anticoagulant was mixed with an equal volume of a Density Centrifugation Medium (DCM) comprising PERCOLL in 0.25 M sucrose at a density of 1.110 g/ml. The mixture was centrifuged at 1600 X g for 20 min.

2) The mononuclear WBCs (MNC) were harvested by pipetting from a band that formed at the meniscus of the equilibrated mixture. The DCM/MNC suspension was mixed with an equal volume of 0.14 M KOH, mixed well and heated at 95°C for 30 min.

3) After cooling to room temperature, the resulting hydrolysate was adjusted to pH 8.0 ± 0.5 by adding one-tenth volume of a solution comprising:

0.65 N acetic acid

0.066 M Tris (hydroxymethyl) aminomethane [Tris base]

0.084 M Tris·HCl

4) Amplification reactions received either 50 µl of this lysate or 50 µl of 1 µg of purified human WBC DNA in 80 mM potassium acetate (Reference System).

Test reactions received the number of pUCHIV templates indicated in Table 3. One negative control reaction was done for each condition (**A-D**) and these values were: 2988, 2179, 5740, and 5602 RLU, respectively.

Table 3

	Standard	Enhanced
	A	**B**
Reference System 5 pUCHIV	12,849 816,241 10,397 722,462 478,359 890,801 615,661 2,608 605,710 89,926	992,207 1,013,207 987,214 916,050 1,004,124 980,016 951,094 1,009,547 988,996 973,544
	C	**D**
Lysate 10 pUCHIV	5,381 5,604 5,568 40,029 4,980 5,245 5,385 4,826 4,937 5,067	844,281 779,576 888,957 850,735 905,316 889,550 611,966 645,488 849,922 797,581

Although the reference system, standard IM2 results (**A**) showed good initiation effectiveness, it is evident that the enhanced system (**B**) is significantly better; all

10 signals are essentially saturated under these conditions. Furthermore, the lysate sample results (**C**, **D**) make it extremely clear how much benefit can be achieved from using the initiation enhancements.

EXAMPLE 5: OUTSIDE PRIMERS

This experiment re-examined the enhancement obtained with each of the outside primers under the challenging conditions of a low target level (3 PUCHIV) in the presence of lysate. All reactions received 1 U *Bst* DNA polymerase and were subjected to the 10 minute incubation at 60°C. The outside primers used (at 3 pmol each per reaction) and the combinations tested are shown in the top row of Table 4. The primer 4312b has the identical sequence complementary to HIV as does T74312 but does not have the promoter sequence.

The RLU values obtained from ten (10) replicate reactions for each condition are shown in Table 4. The bottom row of Table 4 shows the geometric mean of the individual replicate results for that condition. The negative control results for each condition (**A** - **E**) were 776, 2850, 4053, 3875, and 4126 RLU, respectively.

Table 4

Outside Primers				
None	**T74312**	**4312b**	**4312b & 4009**	**T74312 & 4009**
849,178	866,790	929,644	824,030	716,145
3,867	804,203	3,506	4,451	910,595
382,761	4,064	959,438	829,668	880,340
374,433	901,779	895,765	861,299	726,859
284,409	896,920	3,950	240,405	922,937
43,293	850,814	3,892	960,310	866,289
3,893	883,606	3,637	944,199	992,987
3,722	1,083,540	867,171	941,293	925,316
27,165	998,443	858,293	895,293	875,782
893,489	920,823	930,880	909,846	958,355
67,752	**528,996**	**100,820**	**461,481**	**873,055**

As seen previously in Example 2, these results show that the outside promoter-primer T74312 can promote enhanced initiation even in the absence of 4009. Furthermore, these results strongly suggest that the enhancement potential of T74312 benefits from the promoter moiety since the homologous non-promoter-primer, 4312b, did not stimulate initiation significantly over the control condition with no outside primers. One possible mechanism that could account for these results is shown in Figure 3. It is likely that T74312 can initiate a IM2 process by being extended on its complement in the usual way. Note that this step should not interfere with the normal initiation steps primed by the principal promoter-primer, T74116, since they occur on different strands.

Initiation by T74312 in this reaction might be expected to be less efficient than by T74116 since T74312 is present at a lower concentration; however, any T74312 initiations that are successfully completed will result in multiple single-stranded RNAs, which are templates for highly efficient IM3-type initiation by T74116, and which can significantly and preferentially accelerate the accumulation of competent pol1 amplicons during the early stage of the reaction. It probably is desirable for the outside promoter-primer (e.g., T74312) to have lower activity in the reaction than the inside promoter-primer (e.g., T74116) since we have found that highly efficient transcription on both strands can inhibit effective amplification.

Note that different sequences can have different rates of hybridization to their respective complements even at identical concentrations. Therefore the ratio of priming activities for two different oligonucleotide sequences may not be the same as the ratio of their concentrations. However, the molar concentrations are a useful first approximation of the relative activities of two different promoter-primers, and the optimum ratio can be determined by routine experimentation. Furthermore,

methods for quantifying hybridization rates are well known in the art and can be used to resolve apparent anomalies in effective concentrations.

It is evident that 4009 improves initiation effectiveness in addition to any role it may serve in completing the formation of transcriptionally-active species initiated by extension of T74312 (diagrammed in Fig. 3). Not only did the presence of 4009 produce a clear enhancement in this experiment even when paired with the non-promoter-primer, 4312b, but it also promoted enhanced initiation in Example 2 in the absence of either 4312 species.

A possible enhancement mechanism consistent with these observations is shown in Fig. 4. Here, primer 4009 is capable of priming DNA synthesis, which can displace previously synthesized DNA extended from the inside promoter primer, T74116. It is desirable that this outside primer have lower activity than the inside promoter-primer to make it less likely that the outside primer will be extended first, rendering the primary target region double stranded and thus inaccessible to initiation by the inside promoter-primer (e.g., T74116).

EXAMPLE 6: DNA POLYMERASE PROPERTIES

The experiments shown in this example were done to determine if properties other than thermostability of the supplemental DNA polymerase were important to the initiation enhancement mechanisms. The results shown in Table 5 were from three different experiments, each with its own goals, but each contained similar controls which can be compared as references to judge the relative merit of each enzyme. The RLU values in the bottom group, labeled "**None**", were from standard IM2 reactions, incubated with no outside primers and no thermostable DNA polymerase. The middle group, labeled "***Bst*-1**", was treated using the enhanced initiation procedure as described under General Methods and employed *Bst* DNA polymerase from Bio-Rad. The

top group shows the results of the same enhanced initiation procedure substituting one of the alternative thermostable DNA polymerases indicated in the column headings. **"*Bst*-2"** denotes a sample of *Bst* polymerase from a second vendor, Molecular Biology Resources; **"Bca"** corresponds to DNA polymerase from *Bacillus caldotenax* (TaKaRa); **"REPLITHERM™"** is a DNA polymerase available from Epicentre, **"KLENTAQ™"** DNA polymerase (Ab Peptides) is a derivative of *Thermusaquaticus* DNA polymerase as described below. The samples were all handled using the enhanced initiation methods described under General Methods. The respective DNA polymerases indicated were used for the 10 minute, 60°C incubation step. The reactions contained WBC lysates, or were the model reference system and received the average template inoculum shown in Table 5.

Table 5

	Bst-2	Bca	Replith-erm	KlenTaq
Reaction: pUCHIV:	Lysate 5	Model Sys 4	Model Sys 4	Lysate 5
Test Enzyme	1,216,201 1,041,976 952,373 1,039,396 905,270	812,767 801,084 851,248 855,734 811,228 846,270 866,044	842,161 818,499 1,238 866,246 1,262 865,195 1,455	11,564 811,042 153,291 566,063 625,274 427,127
G.M.:	**1,025,760**	**834,568**	**52,998**	**245,204**
***Bst*-1**	547,626 276,219 710,203 19,428 728,553	944,662 913,013 906,523 921,547 954,490 862,586 891,032	944,662 913,013 906,523 921,547 954,490 862,586 891,032	906,711 891,475 654,163 33,052 813,337 899,851
G.M.:	**273,150**	**912,946**	**912,946**	**483,597**
None	3,546 3,207 3,191	855,208 1,259 849,200 1,334 1,342 1,277 1,461	855,208 1,259 849,200 1,334 1,342 1,277 1,461	5,191 5,029 5,337 5,417 5,648 5,579
G.M.:	**3,311**	**8,441**	**8,441**	**5,363**

Table 5 shows that several thermostable DNA polymerases other than *Bst* were also capable of supporting enhanced initiation effectiveness in concert with outside primers. In separate experiments, we found that some other thermostable DNA polymerases did not seem to act synergistically with the nested primers to yield enhanced initiation. These included native DNA polymerases from *Thermus aquaticus* (*Taq*), *Thermus flavus* (Tfl), *Thermus thermophilus* (Tth), *Thermococcus litoralis* (Vent™, New

England Biolabs), or Retrotherm™ (Epicentre). Some of these did confer improved initiation effectiveness compared to standard IM2 if used in an initial 10 minute, 60°C primer extension step in a reaction without outside primers; however, this improvement was never as extensive as the fully enhanced system described above.

The four polymerase enzymes that did support fully enhanced initiation have at least one property in common, lack of a 5'→3' exonuclease activity which may contribute to their effectiveness. *Bst*, Bca and KLENTAQ are each homologs of *E. coli* DNA polymerase I. The 5'→3' exonuclease that is usually found in this class of enzyme is removed by proteolysis during purification of *Bst* by both vendors. Bca and KlenTaq are both manufactured by expression from respective clones of mutant genes defective in this activity. REPLITHERM is reported by its manufacturer to lack any exonuclease activity. That the enhancement mechanism benefits from 5'→3' exonuclease deficiency is suggested here because of this correlation and further corroborated by the superior efficacy of KLENTAQ compared to the native parent form of Taq polymerase.

In these and other experiments, the three polymerases from *Bacillus* species seemed to support more consistent, stable enhancement than either REPLITHERM or KLENTAQ; therefore, these three related enzymes may share a property that distinguishes them from the other two. One possibility is that efficient strand displacement activity, which is known to differ among DNA polymerases, could contribute to mechanisms such as shown in Fig. 4, but other possibilities are not excluded.

These insights showed that the benefits conferred by the fully-enhanced system were not simply dependent on a brief window of elevated primer annealing stringency enabled by the thermostable DNA polymerase, but that the system as configured here has mechanistic advantages, which were not obvious, nor predictable, from prior art.

Example 7: Other Target Regions

The initiation enhancements were also tested and shown to work for target regions other than pol1. The inside target region shown here is called gag11 and uses the promoter-primer T7811 and the non-promoter primer 872. Primer placement for the nested gag11 target region corresponds to Fig. 1 with 780, T7811, 872, and T71062 substituted for 4009, T74116, 4195, and T74312, respectively. The sequences of these primers are:

SEQ. ID NO. 5 (780): 5'-TGCACCAGGCCAGATGAGAGAACCA-3'

SEQ. ID NO. 6 (T7811):

5'-[AATTTAATACGACTCACTATAGGGAGA]AGTGACATAGCAGGAACTA-3'

SEQ. ID NO. 7 (872): 5'-AGATTTCTCCTACTGGGATAGGT-3'

SEQ. ID NO. 8 (T71062):

5'-[AATTTAATACGACTCACTATAGGGAGA]TTGGACCAGCAAGGTTTCTGTC-3'

where the bracketed sequence corresponds to the T7 promoter sequence as described previously (Kacian et al., supra). The promoter portion can be replaced with other functional promoter sequences as described above. The HIV sequences of T7811 and 872 are disclosed in McDonough et al., *supra*.

In this example, 872 was used at 30 pmol/reaction. The principal promoter-primer, T7811, and the outside primers, T71062 and 780, were present at the indicated concentrations. Otherwise, the reactions were handled as described under General Methods using 1 U of *Bst* DNA polymerase per reaction.

All reactions contained 50 μl KOH-hydrolyzed lysate as described in Example 4, and positive reactions received an average of 5 pUCHIV templates. Negative control results for each of these conditions (**A** - **H**) were 5682, 6501, 5775, 4954, 5689, 5140, 5079, and 4805 RLU, respectively.

Table 6

Principal Promoter Primer T7811	Outside Primers (pmol/100 µl)			
	780: 0 T71062: 0	5 0	5 5	5 10
	A	B	C	D
15	8,456 14,254 5,990 31,141 18,771 18,517	6,828 5,873 5,336 35,033 14,259 10,092	86,674 197,119 8885 77,964 76,564 16,734	96,750 51,773 61,521 59,049 41,677 39,743
G.M.:	**14,087**	**10,103**	**49,749**	**55,786**
	E	F	G	H
30	20,083 24,839 6,795 9,958 5,980 10,243	20,020 8,711 15,840 74,433 19,589 19,289	91,414 24,645 36,989 109,757 39,540 47,766	119,865 143,728 75,950 36,031 77,141 15,626
G.M.:	**11,287**	**20,657**	**50,843**	**62,005**

This example shows the benefits of titrating each primer independently. The results of this and other similar experiments are consistent with the expectation, as discussed above, that the optimum concentration of the outside primers should be lower than that of the inside primers. Further optimization improved the gag11 initiation enhancement even more as shown in the data in the next example.

EXAMPLE 8: MULTIPLEX AMPLIFICATION

In some cases it is desirable to amplify two or more distinct target regions in the same reaction. Such "multiplex" amplification reactions, containing 2 or more pairs of primer sets, each delimiting a separate target region, are known in the art. It is most common in such reactions for each target region to amplify less well than

if each target region were amplified in separate reactions. Not only do both (or all) true-target amplicons compete with each other for amplification reaction components, but the potential for non-target initiation and competitive amplification should increase as (about) $p_p \times p_t$, where p_p is the total concentration of all promoter-primers in the reaction and p_t is the total concentration of all primers present (or $\sim p_t^2$ in a case such as routine PCR wherein all the primers are functionally equivalent for initiation).

These complications are a significant impediment to routine development of multiplex amplification systems with reliable detection sensitivity for very low template levels. Nevertheless, using the target-specific initiation enhancements described herein, we have been successful in identifying a multiplex reaction composition with high sensitivity for both pol1 and gag11 targets.

Table 7 summarizes the results of one such experiment. Condition **A** was a standard IM2 procedure in which each of the ten (10) replicate reactions received the pol1 inside primers (T74116 and 4195) and the gag11 primers (T7811 and 872) but no outside primers. After amplification, 50 μl of each reaction was removed and analyzed by hybridization using the pol1 probe. The remaining 50 μl of each reaction was analyzed using the gag11 probe. The results in the pol1 section of column **A** are arrayed in the same sample order as the gag11 results. (i.e., 50 μl of sample #1 yielded 7,448 RLU when analyzed with the pol1 probe; the remaining 50 μl gave 19,596 RLU when analyzed with the gag11 probe.)

Likewise, the RLU values in column **C** reflect analysis of half of each respective reaction using the pol1 or gag11 probes as shown. Condition **C** was the enhanced initiation procedure described under General Methods above except that eight oligonucleotide primers were present (780, T7811, 872, T71062, 4009, T74116, 4195 and T74312,

at 5, 30, 30, 10, 3, 15, 30 and 3 pmol/reaction, respectively).

Condition **B** was the enhanced initiation procedure using only the four pol1 primers, and condition **D** samples received only the four gag11 primers. Each of the replicate reactions in **B** and **D** was analyzed by hybridization using the full reaction volume.

The reactions shown in Table 7 each received an average of 5 pUCHIV templates and 50 μl of lysate prepared as described in Example 4. The corresponding negative controls for these conditions (**A**, **B**, **C**, **A'**, **D**, and **C'**) were 2094, 2907, 2925, 1799, 2014 and 2315, respectively.

Table 7

	Multiplex IM2	**Separate Enhanced**	**Multiplex Enhanced**
	A	**B**	**C**
pol1	7,448	1,150,134	1,078,254
	3,397	1,201,876	1,143,278
	3,314	1,170,546	1,106,627
	3,469	1,160,177	1,112,210
	3,314	1,143,588	1,111,058
	3,226	1,154,678	1,140,999
	3,136	1,153,301	1,118,935
	3,192	1,195,168	3,120
	3,185	1,178,318	1,136,254
	151,392	1,204,093	1,122,474
G.M.:	**5,220**	**1,170,994**	**621,254**
	A'	**D**	**C'**
gag11	19,596	389,763	81,091
	2,009	327,397	92,182
	17,717	212,354	110,175
	2,386	318,371	107,628
	2,065	345,542	74,106
	211,008	280,156	78,950
	26,975	120,927	173,221
	84,759	323,234	2,129
	69,965	167,985	76,044
	145,029	162,030	68,690
G.M.:	**21,018**	**248,238**	**63,089**

It was apparent from the gag11 results with the enhanced system that this target region amplified to a greater extent in a reaction comprising only gag11 primers (**D**) than in a reaction comprising pol1 and gag11 primers (**C'**). Furthermore, it was apparent for both target regions, especially pol1, that detection effectiveness was significantly greater in the enhanced multiplex system (**C**) than for the multiplex IM2 system (**A**). Note that the superior initiation effectiveness of gag11 (**A'**) compared to pol1 (**A**) in standard IM2 is consistent with many previous results.

Baseline signal levels (pol1 RLU=3120, gag11 RLU=2129) were observed in the same enhanced multiplex samples (**C**,**C'**) when analyzed by hybridization with each probe, indicating that there was no HIV DNA in these samples to be amplified. A single failure in 10 replicates is not unexpected at the 5 template input level based on the Poisson distribution ($p \geq 0.065$). Therefore, these results indicate that this multiplex system is able to detect single copies of two different target regions in the same reaction.

Other embodiments are within the following claims.

SEQUENCE LISTING

1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: NUCLEIC ACID AMPLIFICATION

(iii) NUMBER OF SEQUENCES: 8

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(A) MEDIUM TYPE: 3.5" Diskette, 1.44 Mb storage
(B) COMPUTER: MacIntosh Powerbook 140
(C) OPERATING SYSTEM: Apple P.C. DOS (version)
(D) SOFTWARE: Microsoft Word (Version
5.0)

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:

(A) APPLICATION NUMBER:
(B) FILING DATE:

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(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24
(B) TYPE: nucleic
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

ATTCCCTACA ATCCCCAAAG TCAA 24

2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 48
(B) TYPE: nucleic
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) SEQUENCE DESCRIPTION: SEQ ID NO: 2

AATTTAATAC GACTCACTAT AGGGAGACAA ATGGCAGTAT TCATCCACA 39

2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23
(B) TYPE: nucleic
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) SEQUENCE DESCRIPTION: SEQ ID NO: 3

GTTTGTATGT CTGTTGCTAT TAT 23

2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 45
(B) TYPE: nucleic
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) SEQUENCE DESCRIPTION: SEQ ID NO: 4

AATTTAATAC GACTCACTAT AGGGAGACCC TTCACCTTTC CAGAG 45

2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25
(B) TYPE: nucleic
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) SEQUENCE DESCRIPTION: SEQ ID NO: 5

TGCACCAGGC CAGATGAGAG AACCA 25

2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 46
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) SEQUENCE DESCRIPTION: SEQ ID NO: 6

AATTTAATAC GACTCACTAT AGGGAGAAGT GACATAGCAG GAACTA 46

2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) SEQUENCE DESCRIPTION: SEQ ID NO: 7

AGATTTCTCC TACTGGGATA GGT 33

2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 49
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) SEQUENCE DESCRIPTION: SEQ ID NO: 8

AATTTAATAC GACTCACTAT AGGGAGATTG GACCAGCAAG GTTTCTGTC 49

Claims

1. Method for amplification of a nucleic acid strand in a test sample, comprising the steps of:

first contacting said nucleic acid strand from said test sample simultaneously with at least three oligonucleotide primers, at least one primer being a promoter-primer, at least one said primer being complementary to said nucleic acid strand, and at least one other said primer being complementary to a strand complementary to said nucleic acid strand; and

second contacting said nucleic acid strand and primers with one or more proteins having RNA-directed and/or DNA-directed DNA polymerase activities, an RNA polymerase activity and an RNAse H activity, under primer-extension conditions to allow amplification of said nucleic acid strand at constant temperature.

2. The method of claim 1, wherein said nucleic acid strand is a DNA strand, and prior to said second contacting step said nucleic acid strand and primers are contacted at about 60°C or above with an enzyme having DNA polymerase activity active at about 60°C or above.

3. The method of claim 1, wherein said second contacting step is at about 42°C or above in the presence of a reverse transcriptase.

4. The method of claim 1 wherein said nucleic acid strand and said primers are heated to about 95°C or higher prior to said second contacting step.

5. The method of any claims 1-4 wherein four primers are used in said first contacting step.

6. The method of claim 1, wherein one said primer is provided at a concentration different from one other said primer.

7. The method of claim 5, wherein one said primer is provided at a concentration different from one other said primer.

8. The method of claim 5, wherein two said primers are provided at a concentration different from said other primers.

9. The method of claim 1, wherein two said primers are plus-sense primers and the inside plus-sense primer is a promoter-primer.

10. The method of claim 1 wherein two said primers are minus-sense primers and said outside primer is a promoter-primer.

11. The method of claim 1 wherein all said enzyme activities are provided by a reverse transcriptase and an RNA polymerase.

12. The method of claim 11 wherein said enzyme activities are supplemented by an RNAse H having no DNA polymerase activity.

13. The method of claim 2 wherein said DNA polymerase lacks 5'-3' exonuclease activity.

14. The method of claim 13 wherein said DNA polymerase is derived from a DNA polymerase, which in its natural form possesses 5'-3' exonuclease activity.

15. The method of claim 13 or 14, wherein said DNA polymerase is the DNA polymerase I of a *Bacillus* species.

16. The method of claim 15, wherein said species is *Bacillus stearothermophilus* or *Bacillus caldotenax*.

17. The method of claim 1 wherein the two outside primers hybridize to said nucleic acid strand or its complement at most 2000 bases apart.

18. The method of claim 1 wherein the two outside primers hybridize to said nucleic acid strand or its complement at most 500 bases apart.

19. The method of claim 1 wherein the two outside primers hybridize to said nucleic acid strand or its complement at most 350 bases apart.

20. The method of claim 6 wherein one said primer is provided at a concentration between 1 and 10 μM and another said primer is provided at a concentration between 10 and 50 μM.

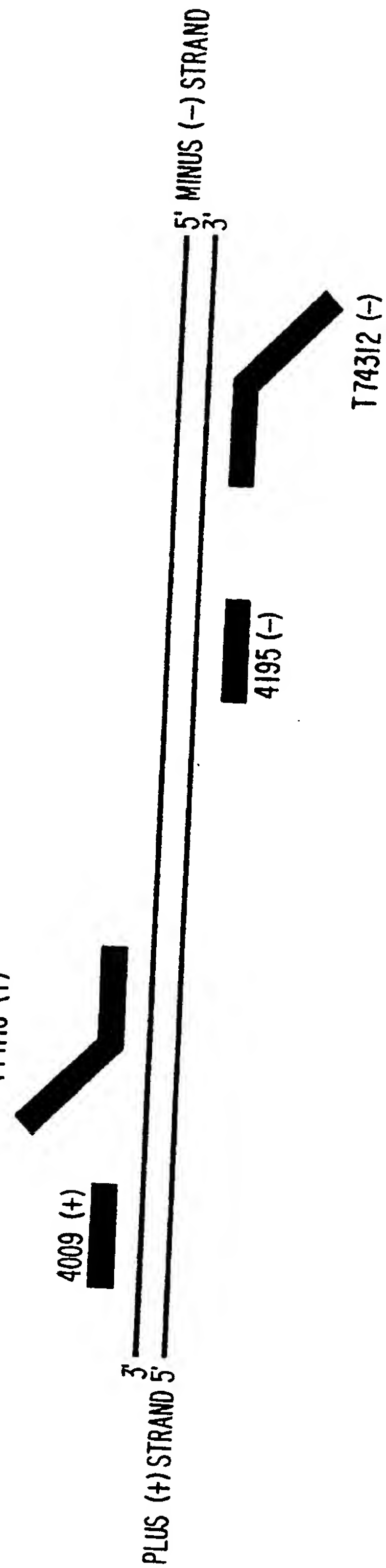


FIG. 1.

FIG. 2.

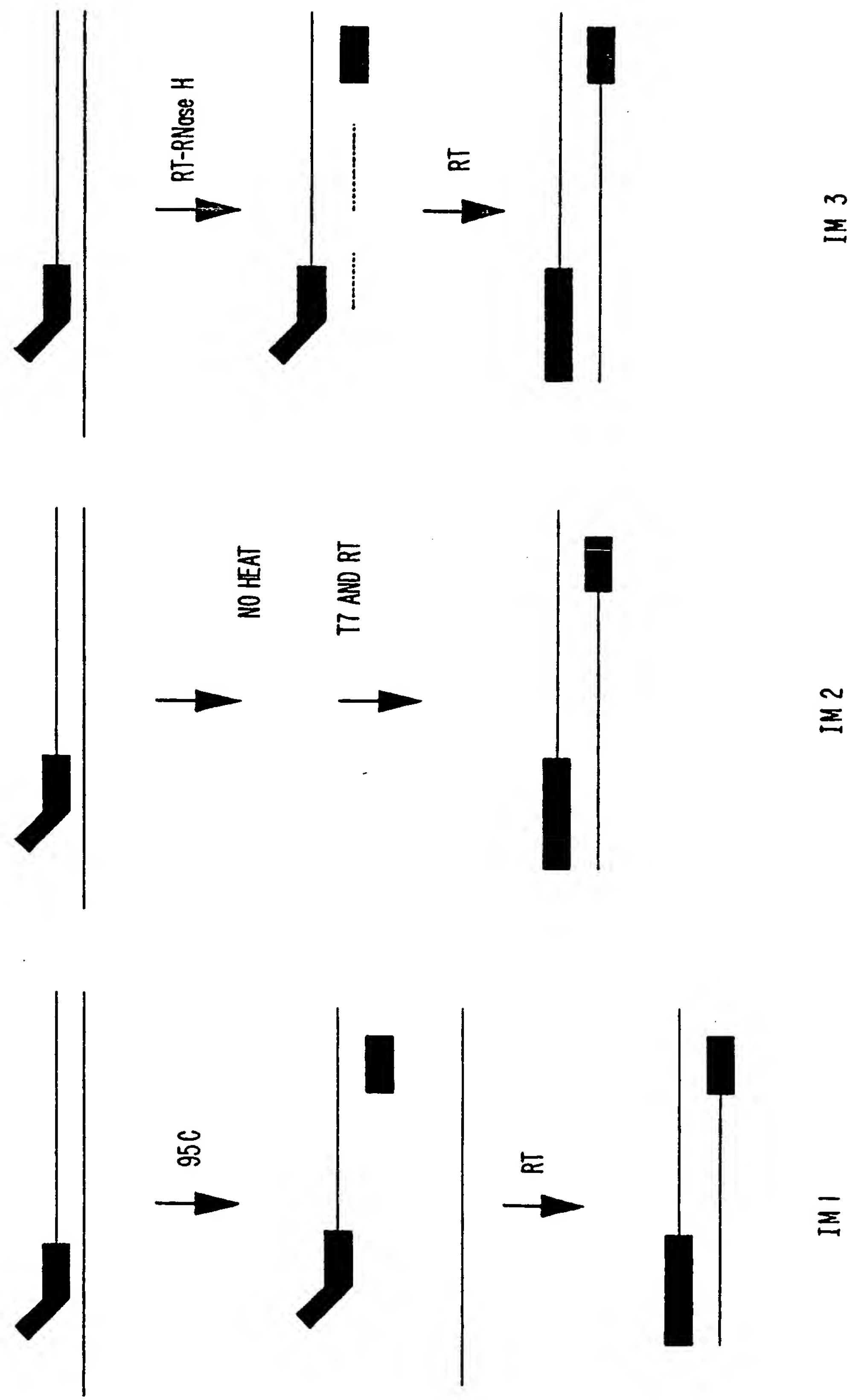
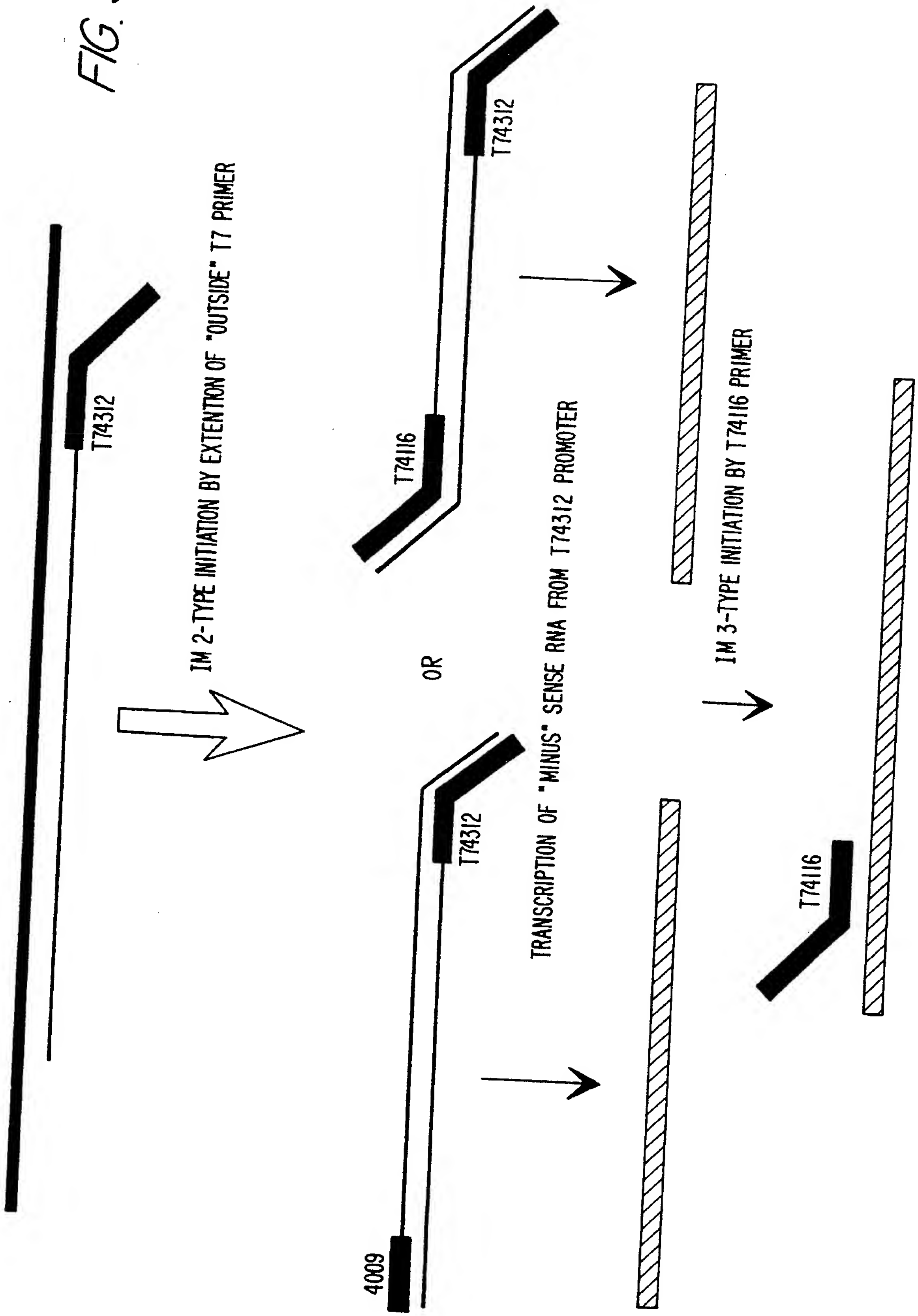


FIG. 3.


T74312
IM 2-TYPE INITIATION BY EXTENTION OF "OUTSIDE" T7 PRIMER
T74116
T74312
OR
T74312
4009
TRANSCRIPTION OF "MINUS" SENSE RNA FROM T74312 PROMOTER
IM 3-TYPE INITIATION BY T74116 PRIMER
T74116

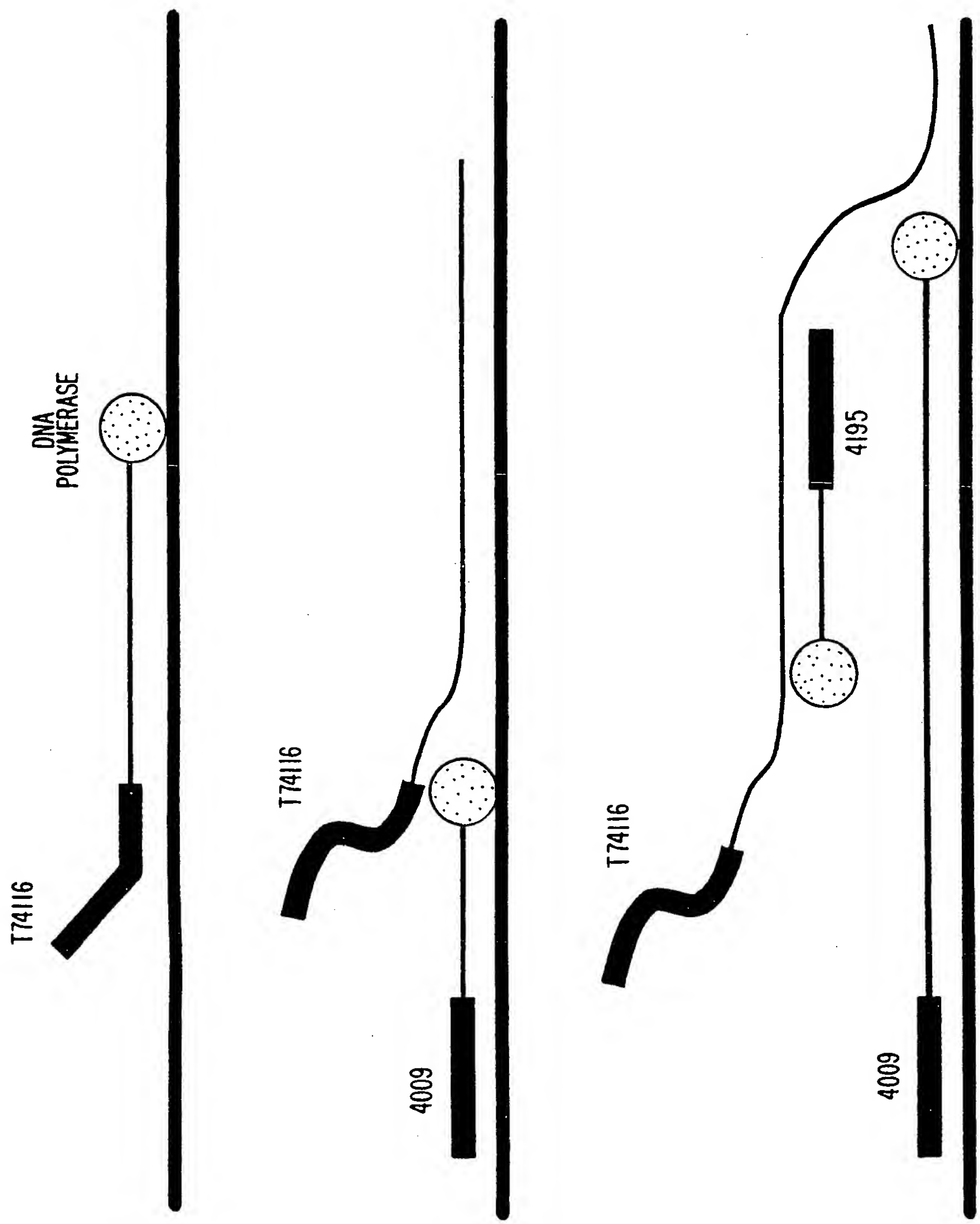


FIG. 4.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 94/08307

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF VIROLOGICAL METHODS, vol.35, 1991, AMSTERDAM NL pages 273 - 286 T.KIEVITS ET AL. cited in the application see page 275; figure 1 see page 280, paragraph 2 ---	1,3-5
X	EP,A,0 545 010 (BOEHRINGER MANNHEIM GMBH) 9 June 1993 see page 5, line 7 - line 48 see page 6, line 13 - line 50 ---	1,3,4,6, 11,12, 17-20

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
9 November 1994	02. 01. 95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	De Kok, A

Internati Application No
PCT/US 94/08307

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	EP,A,0 519 338 (F. HOFFMANN-LA ROCHE AG) 23 December 1992 see page 3, line 7 - line 25 see page 4, line 5 - line 55 see page 5, line 2 - line 30 see page 8, line 11 - line 43 see page 9, line 12 - line 18	1-6, 9-13, 17-19
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P,X	ANALYTICAL BIOCHEMISTRY, vol.217, no.2, March 1994, ORLANDO US pages 248 - 254 T.LIVACHE ET AL. see the whole document	1,2,4,5, 9,10

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